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Glycation of Whey Proteins : Technological and Nutritional Implications

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ABSTRACT

Whey proteins are globular proteins that have received much attention due to their high nutritional value and characteristic functional properties. In addition to being part of the protein system in milk, they constitute the main proteins in whey and whey protein products. Interaction of whey proteins with reducing sugars and carbohydrates via Maillard reaction have been extensively studied in milk and in model systems. Glycation of individual whey proteins results in variable increases in their solubility, thermal stability, antioxidant activity, and emulsion and foam stabilization. Limited glycation of whey protein products particularly whey protein isolates (WPI) using polysaccharides has been studied with the aim to produce conjugates with modified functional properties and acceptable sensory properties. An overview is presented here on the effect of glycation on individual whey proteins and whey protein products and the potential uses of the glycated whey proteins.

Key Words : Whey proteins; Maillard reaction; Functional properties; Nutritional value

1-Introduction

Thermal treatment is a basic step in the processing of milk and milk products in order to control the safety and quality of the obtained products. The severity of the thermal processing ranges from the mild thermization applied directly to raw milk at the dairy farm up to sterilization of evaporated and condensed milk. Several chemical changes usually occur in thermally treated milk [1] resulting in variable losses in vitamins and enzymes, denaturation of milk proteins and interaction between lactose and milk proteins to form protein-sugar conjugates.

The interaction between lactose and milk proteins has received special interest since the early studies on milk processing, whereas it has diversified effects on the digestibility, and nutritional, functional and conformational changes in milk proteins as well as the quality of dairy products. During storage, further changes occur in the formed lactose-milk protein conjugate depending on the storage conditions.

Over the years research [2,3,4] has been extended to study conjugation of milk protein with various carbohydrates aiming for better understanding the mechanism of protein-carbohydrate conjugation and to produce milk protein products of modified functionality and antigenicity.

Whey proteins are a group of globular proteins representing 20% of the total proteins of bovine milk and almost the total proteins of whey [5]. The main proteins in whey and their concentration (relative to the total whey proteins) are β-lactoglobulin (~ 55%), α-lactalbumin (~ 20%), blood serum albumin (~7%), immunoglobulins (~13%) and minor

proteins (~ 5%). In addition, whey obtained from cheese manufacture contains the casein glycomacropeptide (GMP) which arises from the action of chymosin on κ-casein.

There has been a global increase in the production and utilization of whey protein products due to the increase in cheese production and concentration of whey. An annual increase in cheese production of 2.6% has been reported by IDF [6]. Also, the advent of powerful industrial technologies for the separation of whey proteins based on membrane and ion exchange methods has enabled the dairy industry to produce whey protein products of tailored composition and functionalities. It has been estimated that the manufacture of whey protein products can increase the revenue of a cheese factory by 11%. The world-wide market [7] value for whey powders and protein ingredients amounted to US$ 9.8 billion in 2013 and has been projected to reach a value of $11.7 billion in 2017 [8]. Of the 31 million tons of the dried whey products produced in the USA, about one fifth has been whey protein isolates [9]. The demand for whey proteins has been boosted by the consumer awareness on its impact of food on human health, the documented nutritional and health properties of whey proteins [10], and consumer demand for high-protein foods and supplements. Whey protein products are widely used food ingredients due to their broad range of functionality [11], along with their exceptional nutritional and physiological properties. Whey proteins are used in several food applications, including protein supplements, baked goods, dairy, and meat products, and sports drinks. However, the heat-induced changes in the functionalities of whey protein limit its use in some application such as beverages. Several methods have been proposed to modify the functional properties of whey protein products, including partial enzymatic hydrolysis and glycation.

With the advent of more sensitive tools for in depth monitoring of the formation of carbohydrate-whey protein conjugates, detailed information has begun to accumulate for better understanding the mechanism, and consequences of these reactions. Also, the effect of glycation on the functional properties of milk proteins, particularly whey proteins has been extensively studied with the aim to prepare whey protein products having tailored functional properties. This article is a comprehensive overview on the effects of glycation on the technological and nutritional properties of whey proteins.

2-Maillard reaction

The Maillard reaction [12] involves a very complex series of reactions occurring at the same time to proteins and carbohydrates during heating and/or storage. The reaction is very difficult to control because several factors are involved. Hodge [13] elaborated a comprehensive scheme of for the Maillard reaction which can be divided into three parts: early, intermediate, and advanced stages [14] as summarized in Fig 1.

In the early stage of the Maillard reaction condensation reactions occurs between the carbonyl groups of reducing sugars (such as glucose and lactose) and mainly the ε-amino group of lysine, or to a lesser extent the imidazole and indole groups from histidine and tryptophan, respectively, and after the α-amino groups of terminal amino acids in proteins/peptides. This results in the formation of unstable Schiff base components with the release of water. A Schiff base undergoes cyclization to N-substituted glycosylamine, which then forms an irreversible Amadori rearrangement product (ARP), 1-amino-1-deoxy-2-ketose. The products of the early stage are colorless, which don't exhibit any absorption in the ultraviolet region.

In the intermediate stage, the Amadori rearrangement products undergo degradation by a number of different reactions depending on the pH. At pH ≤ 7 it undergoes 1,2-enolization mainly with he formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved). At pH > 7 it undergoes 2,3-enolization where reductones such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMFone) are formed. In both cases fission products (short-chain carbonyl compounds such as methyl glyoxal) are formed. These products are highly reactive leading to the formation of the advanced stage end products of Millard reaction. The products of the intermediate stage are colorless or pale yellow with strong absorption in the ultraviolet region.

In advanced stage of Maillard reaction complexes, and variable compounds are formed depending on the reaction conditions. Reactions involving variable pathways occur, including cyclization, dehydration, retro-aldolization, enolization, oxidation, fragmentation, acid hydrolysis, isomerization, rearrangement, free radical reactions, and further condensation, leading to the formation of a large number of poorly characterized compounds denoted as advanced Maillard reaction products (AMP). Polymerization reactions lead to the formation colored nitrogenous polymers and co-polymers, known by the generic name of melanoidins. They have been reported to exhibit several biological activities but the structure of only very few melanoidins has been elucidated [15].

The rate and degree of glycation are influenced by several factors [3] including the type carbohydrate used, the protein/carbohydrate ratio, and reaction conditions (dry/wet, temperature/time, pH). Differences in the effect of the sugars used have been attributed to the rate of their transformation from the ring form (non-reactive) to the chain form

(reactive). Sugars which exhibit rapid opening of the ring form induce faster and higher degree of glycation [2]. Also, the high molecular weight and negatively charged saccharides induce glycation at a slower rate than neutral mono, di- and oligosaccharides [16]. This has been attributed to the steric hindrance of the bulky and charged saccharides. The degree of glycation can be controlled by changing the protein/ carbohydrate ratio whereas limited glycation can achieved with the use of high protein/ carbohydrate ratio. Also, alkaline pH (e.g. pH 9) enhances the reactivity of the reactants leading to high rate of glycation between chains form of the sugar and the un-protenated ε-amino group of proteins and peptides [2]. Increasing temperature and heating time generally increase the rate and degree of glycation [3]. In addition, icreasing the relative humidity during dry heating enhances Maillard reaction due to increased diffusion of the reactants. However, slower Maillard reaction occurs in aqueous solution than in dry heating due to the retarded formation of water molecules during Amadori rearrangement [3]. In dry heating the most influential factors affecting the glycation of β-lactoglobulin (β-Lg) with dextran were the temperature and water activity (a_W) [17]. The ability of chlorogenic acid, the characteristic coffee plant phenol, to block the formation of advanced glycation end products (AGE) has been recently reported [18]. Thus the addition of 50m M of chlorogenic acid to the reaction medium of β-Lg and glucose as a reducing sugar in 30% aqueous ethanol heated at 70^{O}C prevented the formation of AGE as detected using ELISA. This has been attributed to the electrostatic binding of chlorogenic acid to the reactive lysine sites of β-lactoglobulin.

3. Analytical approaches used in glycation studies

Many protocols have been developed to follow glycation of whey proteins in simple models systems and in complex systems such as processed milk and milk products. Direct and indirect methods of variable complexity have been followed in the different approaches.

3.1. Identification of the glycation sites. Proteomic and mass spectrometry have been used extensively for the identification of the glycation sites [19, 20, 21]. The proteomic approaches are based on three steps: tryptic digestion of the glycated protein, separation of peptides by chromatographic or electrophoretic techniques followed by identification of the separated peptides by mass spectrometry analysis.

3.2. Determination of losses in chemically reactive lysine. Determination of the residual ε-amino group before, and after Maillard reaction indicates the number of ε-amino group involved in the reaction which can be used as an index for the severity of the thermal treatment. Several methods based on different reagents have been developed to determin the ε-amino groups including 1-fluoro-2,4-dinitrobenzene (FDNB), trinitrobenzenesulfonic acid (TNBS), guanidination, sodium borohydride, o-phthaldialdehyde (OPA) and dye-binding methods [22]. The OPA method was reported to be a simple, rapid, and sensitive method that could be used as a good indicator of the early stage of glycation of whey proteins with mono and disaccharides [4].

3.3. Determination of Maillard reaction products.

3.3.1. Products of the early stage of the reaction. Furosine (*ε-N*-(2 furoylmethyl)-l-lysine) is a non-native amino acid formed from the acid hydrolysis of lactolosyllysine. It has been used as an index for the heat treatment of food products since its discovery in

1966. Determination of furosine has been adapted as a standard method for the determination of heat treatment of milk [23]. The method is based on acid hydrolysis using 6 N HCl followed by ion-pair reverse-phase high performance liquid chromatography. Also, direct measurement of the absorbance of Shiff base at 304 nm has been used as an index for early stage of Maillard reaction [24].

3.3.2. Products of the intermediate stage of the reaction. High pressure liquid chromatography methods have been used to follow the intermediate reaction products such as 5-hydroxymethyl furfural [25].

3.3.3. Products of the advanced stage of the reaction. The advanced stages of Maillard reaction are accompanied with the formation of brown colored compounds. Colorimetric measurement of the brown color has been taken as an index for the advanced stage of this reaction [14]. Also, the fluorescence (λexc 350 nm, λem 440 nm) of milk fraction soluble at pH 4.6 has been used to measure the advanced Maillard products in heated milk [26]. Carboxymethyl-lysine (CML) is among the compounds formed in severely heated products in which the furosine levels have already peaked. CML is an inert compound which received wide interest [27] as index of advanced glycation end products (AGEs). Various instrumental and immunochemical methods have been used [27] to measure CML content including reversed-phase HPLC after drivatization with o-phthalaldehyde (OPA), gas chromatography coupled with mass spectrometry (GC-MS) following methylation of its carboxylic groups, and acylation of its amine groups. Also, the volatile compounds which arise from the Strecker degradation have been used to follow AGEs in UHT milk using dynamic headspace gas chromatography-mass spectrometry [28].

4. Glycation of whey proteins

4.1. β-lactoglobulin(β-Lg)

4.1.1. Degree of glycation and glycation sites

Glycation of β-Lg has been extensively studied as the major whey protein which undergoes heat denaturation, and conformational changes under moderately heat treatments. Also, the implication of β-Lg in cow milk allergy (CMA), whereas about 82% of CMA patients are sensitive to β-Lg, encouraged studies on technologies capable to decrease allerginicity of β-Lg particularly the heat treatments.

Variable numbers, and locations of lacosylation sites in β-Lg have been reported in the different studies. Using proteomic mapping, LC–ESI–MS and tandem mass spectrometric analysis [29], Lys 47 was identified as the first β-Lg lactose-binding site to be detected. Further studies using LC–ESI–MS analysis indicated that lactosylation was involved in nine Lys residues widely distributed within the β-Lg structure, but no modification was detected in Lys 8, 60, 83, 101, 135 and 141 [30]. Lys47 and Lys69 have been identified as the early glycation sites in β-Lg [31]. Milkovska-Stamenova and Hoffmann [21] found that lactosylation occured in β-Lg isolated from pasteurized, UHT milks and infant formulation at Lys 47, 60, 69, 70, 75, 77, 83, 91, 100, 135, 138 and 141 respectively in addition to Lys 101 in infant formulation only which received the highest heat treatment. Proteomic analysis revealed four distinct lactosylation sites (Lys 63,107, 116 and 154) and four carboxymethylation sites (Lys 107, 116, 151 and 157) depending on the heating conditions [32]. Lys 154 was found to be the most sensitive site for modification. Fogliano *et al.* [33] was first to isolate mono-lactosyl-β-Lg from UHT milk

and di-lactosyl-β-Lg from sterilized milk. Meltretter *et al*., [34] detected lactosyllysine in β-Lg isolated from heated milk at Lys 47, 138 or 141. Glycation at Lys 69 was reported to be limited to B variant of β-Lg [35].

The use of different saccharides resulted in the variable degrees of glycation, and number of glycation sites. Glycation of β-Lg was found to increase with the increase of the molecular size and negative charge of the reducing sugar used [16]. Also, difference in the molecular size of the used saccharide resulted in variable glycation at Lys 8, 70 and 75, while the negative charge led to dissimilarity of glycation at Lys 77 of β-Lg [16]. Heating of β-Lg with glacto-oligosaccharides resulted in glycation at Lys 14, 47, 75, 77, 83, 91, 100, 135, and 138 [36]. D-Psicose, is a rare ketohexose that received attention as low calorie sugar with no glycemic effect, enhances weight losses, and exhibits prebiotic effect [37]. It is an epimer of D-fructose with isomerization at C-3 position. β-Lg was glycated with D-psicose and D-fructose at molar protein: sugar ratio of 1:13 by dry heating at 50°C and 55% RH for 48 h [38]. D-Piscose induced more changes than D-fructose especially after the initial stage of Maillard reaction. In addition, glycation of β-Lg with D-piscose resulted in more polymeric compounds, higher antioxidant activity, and lower thermal stability than with the use of D-fructose.

Differences in the glycation of β-Lg can be attributed mainly to the medium (model, milk and milk products) heating conditions (Temperature/time, Dry/wet). Generally, glycation of β-Lg go faster under dry conditions due the increased concentration of the reactants, and absence of water which retards the formation of Amadori rearrangement products.

4.1.2. Other induced changes

Methyl sulfoxide was detected at Met 7, 24 and 145 in β-Lg isolated from heated milk, and in severely processed products Met 7 and 24 were highly modified [34]. Also, during heat of milk β-Lg was susceptible to redox modification including oxidation products of Met 23 and Met161, oxidation of Cys82, Cys122, Cys135, Cys137, and Cys176 to cystic acid and a range of different oxidative modifications in Trp35 and Trp77[32] The side chains of several amino acids undergo carbonylation in heated milk. Mapping the carbonylation sites in several heat treated milk and milk products revealed that β-Lg underwent carbonylation at Lys 60 and 69 and Cys 66, 106 and 160 [21].

4.1.3. Structural changes

Glycation induces variable structural changes in β-Lg compared to heated β-Lg [39]. The degree of unfolding and aggregation was affected by the sugar used and the thermal treatment whereas lactose induced less conformational changes in glycated β-Lg than glucose. More conformational changes of β-Lg were found after conjugation with nystose than with fructofuranosyl nystose, including quenching of fluorescence intensity, the red-shift of fluorescence spectra and the increase in sulfhydryl content [40]. Minor changes seems to occur in the secondary structure of glycated β-Lg. Sonication of β-Lg in the presence of sugars resulted in a slight decrease in α-helix and slight increase in random coil of glycated β-Lg [41]. Concerning the tertiary structure, glycated β-Lg possessed newly exposed hydrophobic patches on the protein surface particularly in the presence of ribose [41]. Czerwenka *et al.*[42] observed an increase in hydrophobicity, and slight conformational changes toward more compact structure in thermally lactosylated β-Lg. Conjugation of β-Lg with fructooligosaccharide (FOS) by dry heating

resulted in partial unfolding of β-Lg, an increase in the β-strand, quenching of the fluorescence intensity and the red-shift of the fluorescence [43]. Circular dichorism indicated alteration in the secondary structure of β-Lg subjected to microfluidization followed by glycation with galactose [44].

4.1.4. Antigenicity

β-Lactoglobulin is known to be a principle factor in cow milk allergy (CMA). Three major β-Lg allergenic epitopes have been identified as fragments of the amino acid residues AA102–124, AA41–60 and AA149–162 respectively [45]. The AA41–60 and AA102–124 are from the loops between β-strands C and D, which are stabilized by either hydrogen or disulfide bonds while fragment AA149-162 are buried in the native structure of β-Lg, inaccessible to IgE and IgG antibodies unless the β-Lg is denatured. The effect of glycation on the antigenicity of β-Lg has been the subject of several studies. In the early stages of Maillard reaction only a small effect was found on the recognition of glycated β-Lg by IgE of sera from CMA patients, whereas a clear masking effect on the recognition epitope occurred by the highly glycated β-Lg [46]. This has been attributed to the presence of Lys residues in β-Lg epitope, and glycation of these residues weaken or prevent binding to IgE. Bovine β-Lg was conjugated with alginic acid oligosaccharide (ALGO) (1:6) and phosphoryl oligosaccharides (POs) (1:8) by the Maillard reaction [47]. The conformation around 8Lys−19Trp was found to be changed in the conjugate. The anti-β-LG antibody response was markedly reduced after immunization with both conjugates in BALB/c, C57BL/6, and C3H/He mice. Reduction of the antibody response was observed in the vicinity of the carbohydrate-binding sites. The ratio of β-Lg/sugar

used was found to affect the antigenicity of β-Lg-fructooligosaccharide (FOS) conjugate [43]. At a ratio of 1:4 a 7 time decrease in antigenicity was found compared to control β-Lg whereas at <1:4 ratio the formed conjugate increased antigenicity depending on the concentration of FOS used. Subjecting β-Lg to microfluidization followed by glycation with galactooligosaccharide (GOS) resulted in a decrease in the antigenicity of the formed conjugate and the lowest antigenicity was achieved at <120 MPa but at higher pressure (160 MPa) the antigenicity increased [48]. Similar results were obtained for glycation of β-Lg with galactose subsequent microfluidization [44].

4.1.5. Functional properties.

The effects of glycation on the functional properties of proteins are important for the development of ingredient with modified and controlled properties. Table 1 summarizes the changes in the functional properties of β-Lg subjected to Maillard reaction under different heating conditions using different carbohydrates. Generally the changes in the functional properties of β-Lg depend on the conformational changes occurred in the glycated β-Lg. The solubility and heat stability of glycated β-Lg increased compared to the native protein which can be attributed to decreased aggregation of glycated β-Lg and shifts in the pI of β-Lg to lower values. β-Lg was found to form fibrils on heating at neutral pH, but in the presence of glucose the formation of these fibrils slowed down and mixture of short fibrils and polydisperse aggregates were formed [66]. The rigidity and thickness of the formed films from glycated β-Lg at air and water interfaces increase with the increase of the molecular weight of the saccharide used in glycation which improve the foams and emulsions stabilities [54]. The reaction conditions (dry/wet,

temperature/time and pH) play an important role in modifying the functional properties of β-Lg through its effect on the conformational changes in β-Lg.

4.1.6. Digestibility and microbial growth

Controversial results have been reported with respect to the digestibility of glycated β-Lg. Chevalier *et al.* [67] reported a decrease in the tryptic digestibility of β-Lg with the increase in glycation with glucose until hydrolysis was stopped after 264 h of glycosylation. Similarly, glycation of β-Lg with glucose [68] and galactose and tagtose [69] reduced the *in vitro* gastrointestinal digestibility of β-Lg which has been attributed to the increased resistance of the peptide bonds to hydrolysis. Contrary to these findings, Morgan *et al.* [70] and Wang and Isamil [71] reported that glycation enhanced the proteolysis of glycated β-Lg. Differences in the conditions (wet/dry) and stages of Maillard reaction (early/advanced), and the conformational change of the protein occurred upon conjugation with the carbohydrate may be responsible for this discrepancy. Conjugates of β-Lg with galactose and lactose were prepared by dry heating, and subjected to *in vitro* digestion with gastrointestinal fluid [72]. Lactobacilli and Bifidobacterium were able to grow on the digest of β-Lg conjugates while they were not able to grow on the digest of β-Lg. The growth of these two species of bacteria on digests of β-Lg conjugates was strain dependent. This suggests that new prebiotic can be prepared from β-Lg by conjugation with lactose and galactose.

4.2. α- Lactalbumin (α-La)

4.2.1. Degree of glycation and glycation sites

Compared to β-Lg, α-La behaved differently in Maillard reaction because of its low molecular weight and compact structure. The rate of reaction was reported to be faster,

and more pronounced in α-La compared β-Lg [73] and that the presence of Ca had no effect on the glycation of α-La. Dry heating of α-La in the presence of glucose at 60°C and relative humidity of 65% for 8 h resulted in glycated α-La with 6-13 glucose units [74]. The rate of Maillard reaction of α-La was found to decrease with the increase of the size of the saccharide used [75]. The degree of glycosylation of α-La with lactose was higher (85%) compared to maltodextrin (31%) by dry heating at 60°C, and a_W of 0.58 for 48 h [75]. Conjugation of α-La with charged saccharides was limited to specific number of molecules due to repulsive electrostatic force [74]. In addition to conjugation with the saccharides, α-La underwent cross-linking during Maillard reaction [76] and the rate of the two reactions was dependent of the saccharide used. Glycation by arabinose and xylose led to a very fast protein cross-linking, whereas relatively low protein cross-linking was obtained with the use of glucose. Lactosylation of α-La by dry heating followed 2nd order kinetic reaction with increased activation energy, and rate constant as a result to increase in temperature and a_W (between 0.33 and 0.44) but decreased on further increase of a_W to 0.58 [75]. In aqueous solution, enhanced lactsylation of α-La was obtained with the increase of pH from 6.0 to 7.9, and the effect of temperature on lactosylation was more pronounced at the low pH [77]. Formation of lactosylamine was found to be the rate-determining step for the reaction between lactose and protonated α-La, while the water elimination step, in the formation of the Schiff base, became the rate-determining step for the un-protonated α-La [77]. lactosylation of α-La was more rapid in whole milk compared to the reaction in aqueous model systems suggesting that the presence of other components of milk affected the reaction [42].

Out of the12 Lys residues in α-La, nine (Lys 5, 13, 62, 93,94,98, 108, 114 122) were found to be lactosylated in UHT, and infant formula [20], while lactosylation at Lys 62 and 108 was not detected in α-La of pasteurized milk. Lys 98 in α-La was the only residue able to form conjugate with hexose in pasteurized and UHT, but more hexose binding sites were found in α-La of infant formula [78].

4.2.3. Structural changes

Circular dichorism (CD) showed no changes in the secondary, tertiary, and quaternary structure of α-La after glycosylation with D-glucose (0-63% degree of glycation) [79] or 21.4% modified Lys residues after glycation with maltopentose at 50 °C and 65% RH for 3 d [80]. In addition, >90 % of α-La remained in the monomeric form under these conditions. The fluorescence intensity of α-La decreased markedly and a shift of λ_{max} to 319 nm were found after conjugation with dextran [17].

4.2.4. Antigenicity

The reported IgG epitopes of α-La are AA 7-18, AA 51-61 and AA 89-108 include 8 of the 12 Lys residues found in α-La. Therefore, it is expected that glycation would decrease allergenicity of α-La. Bu *et al.* [81] found that dry heating (40-60°C, 75% RH) of WPI-glucose mixture to reduce the antigenicity of α-La and the lowest antigenicity of glycosylated α-La was obtained by heating at 52.8°C/78 h for mixture (5.98:1) of WPI/ glucose. Dry heating at 50°C and 65% RH of mixtures of α-La with glucose, maltose and maltooligosaccharides reduced the antigenicity of the protein [82]. Generally the degree of glycation and antigenicity decreased with the increase of the size of the saccharide used, but the highest reduction of antigenicity was observed in maltotriose glycated α-La.

Glycation of Lys 58 was dominant in glucose-α-La and maltotriose-α-La showing the greatest antigenicity reductions. The antigenicity of α-La was greatly reduced by glycation with maltopentose which has been attributed to the shielding of the linear epitopes with the carbohydrate moiety [80]. Also, glycation with maltopentose significantly enhanced the suppressive effect of α-La on lipopolysaccharide stimulated T.HP-1 cells to produce pro-inflammatory cytokines such as IL-6 and tumor necrosis factor-α [80].

4.2.5. Enzymatic digestion

α-La and D-glucose conjugates with different degrees of glycation (0-63%) were prepared [79]. Hydrolysis of these preparations using bovine and porcine trypsin decreased linearly from the untreated α-La up to 65% for maximally glycated α-La indicating that trypsin cannot hydrolyze glycated Lys sites. Hydrolysis of glycated α-La with *Bacillus licheniformis* protease and subtilisin A was independent of glycation, while α-chymotrypsin cannot hydrolyze glycated cleavage sites. Hydrolysis of glycated α-La with non-lysine/arginine specific proteases was found to be dependent on the enzyme sensitivity towards modifications on the binding sites [79]. Extending the dry heating time from 12 h at 60°C/79% RH to 24 h resulted in different rate for the proteolysis of fructose- α-La conjugate [83]. Retarded proteolysis by simulated *in vitro* infant gastrointestinal fluid was found for fructose- α-La conjugate after 12 h heating whereas accelerated proteolysis was observed in case of the 24 h conjugates. Less pronounced effect was observed when fructose- α-La conjugate was subjected to proteolysis using simulated adults gastrointestinal fluid. It has been suggested that glycation of α-La

fructose could increase the bioaccessability of ingested peptides in adults and more pronounced in infant to confer its health benefits [83].

4.2.6. Functional properties.

Limited studies have reported the changes in the functional properties of α-La glycated with different carbohydrates (Table 2). Generally these studies pointed out that the solubility, emulsifying properties, foam stability, thermostability and antioxidant activity of α-La increased by glycation. The role of the type of carbohydrate and changes in the heating conditions on glycation of α-La particularly in aqueous solutions need more studies.

5-Glycated whey protein concentrates (WPC) and isolates (WPI).

5.1. Functional properties

Whey protein concentrates (WPC) and isolates (WPI) have been used for the preparation of glycated whey proteins instead of the individual whey protein for economic and technical reasons. Both WPC and WPI are produced on industrial scale at reasonable cost compared to the purified proteins. The difference between these two products lies in their protein, and lactose contents but both contain mainly the two major whey proteins i.e. β-Lg and α-La in the same ratio to their presence in whey. The high protein content (>90%) and low lactose of WPI favor its use for the preparation of glycated whey protein products as a way for expanding the utilization of whey protein products. The low lactose content of WPI would decrease its interference in WPI glycation with other saccharides. The presence of traces of lactose had no significant effect on the glycation of WPI with maltodextrin [86].

Limited glycation of WPI with polysaccharides gave products of superior functional quality than those prepared by glycation with sugars. Compared to lactosylated WPC WPC-dextran conjugate prepared under the same condition (dry heating of acidified WPC at 100ºC/2h) was characterized by lighter color, high water holding capacity (6 times) and 2-3 increase in emulsion stability [87]. Oligomeric (20,000–200,000 g/mol) and polymeric (>200,000 g/mol) glycated products were obtained by Maillard reaction between glucose, lactose, pectin, and dextran, and WPI [88]. Generally all glycated WPI gave highly viscous solutions, and exhibited increased antioxidant capacity compared to the unmodified protein. The WPI/dextran products exhibited improved heat stability, increased overrum and increased emulsifying activity.

Several studies used dextran and other polysaccharides to achieve the modification of the functional properties of WPI without noticeable effect on its sensory properties, and nutritive value.

Dextran is a neutral polysaccharide composed mainly from linear chains of glucosyl residues linked via α (1→6) glucosidic bond. It is widely used to glycate proteins due to its neutral but reducing nature which avoids the formation of electrostatic complexes with proteins. Conjugated WPI-dextran (DX) was prepared from different WPI: DX ratios by dry heating at 80ºC and 79% RH for 2 h [89]. Conjugate prepared from WPI: DX (1:3) exhibited better emulsion stability than native WPI, and gum Arabic. WPI-DX conjugate was prepared by incubating aqueous solutions containing 10% WPI and 30% DX at pH 6.5, and 60°C for 48 h followed by removal the residual un-reacted DX by ion exchange chromatography [90]. The purified conjugate was characterized by significantly higher stability at 80 °C for 30 min, over pH 3.2-7.5 and ionic strength 0.05-0.2 M, and

emulsifying ability and emulsion stability compared to WPI. Conjugation with DX modified the rheological properties of WPI during the heat gelation although it retained the formation of typical elastic gel [91]. The elastic modulus (G') of the WPI-DX conjugate was one tenth that of the WPI gel and the gel stiffness was greatly reduced. Glycation of WPI with a sufficient number of maltodextrins prevented the protein aggregation before and after heating at 88°C for 2 min at pH 3.0–7.0 and 0–150 mM NaCl or $CaCl_2$ [92]. Steric hindrance has been suggested to be the major mechanism responsible for transparent dispersions of heated glycated WPI with structures smaller than 12 nm. The molecular weight of DX used in glycation affected the degree of loss in the amino groups and the color of the resultant WPI-DX conjugate. The high molecular weight and the low concentration of the DX used gave WPI-DX conjugate of lighter color and less amino group losses. Using uniaxial compression and stress showed that Maillard reaction significantly modified the mechanical properties of WPI-DX gels, and even prevented fracture when conjugate gels were subjected to 80% deformation [93, 94]. Limited glycation, to the early stage of Maillard reaction, was used to improve the solubility and thermal stability of whey protein, without any loss in its nutritional value or digestibility [71]. Mixture (1:4) of WPI: DX was heated at 60^0C and a_w of 0.49 for 96 h gave the highest conjugated product without noticeable color formation. The WPI-DX conjugate showed ~85% increase in solubility compared to WPI at pH 4.5 and protein concentration of 5%, glycated WPI when both were heated at 80 °C for 30 min. The pI of WPI (pH 4.86) was shifted to 4.2 for glycated WPI and this was attributed to the blockage of some amino groups protonated at lower than the pKa values. Also, there was a decrease in surface hydrophobicity of the glycated whey protein, which was attributed

to blockage of surface hydrophobic regions and attachment of a hydrophilic component. Analysis by Raman spectroscopy revealed that the limited glycation of WPI with DX resulted in rigid protein structure resisting unfolding, and exposure of the hydrophobic interior [95].

In order to exploit the industrial uses of WPI-DX conjugate there was a need to separate and concentrate from the reaction medium. Bund *et al.* [96] developed a large scale cation exchange chromatographic method for the separation, and purification β-Lg-DX conjugate. After the removal of un-reacted β-Lg by acid precipitation, and centrifugation, β-Lg-DX conjugate was recovered by chromatographic separation using MacroCap^{TM} SP resin. The glycated protein was relatively pure, containing only traces of β-Lg, and it was heterogeneous due to oligo-glycation. The potential uses of this method for the preparation of WPI-DX need to be studied.

Maltodextrins are starch hydrolysis polymers used extensively in the food industry as stabilizers, thickners, bulking agents and energy sources. They have chains of variable lengths from D-glucose units linked by α-(1→4) and α-(1→6) glycosidic linkages. They are characterized by their dextrose equivalent (DE) value which is a measure of its total reducing power. Pre-acidification of mixed WPI- maltodextrin (1:1) before dry heating at 80º C/65% RH for 1 up to 4 h was found to affect the functional properties of the obtained conjugates [97]. Compared to other treatments conjugate prepared at pH 6 and heating for 2 h exhibited the highest degrees of glycation and thermostability, lowest surface hydrophobicity and lowest isoelectric point. Controversial results were reported for the effect of WPI-maltodextrin ratio on the color of the obtained conjugates [98, 99]. Conjugates were prepared from 1:1 and 1:2 protein: polysaccharide ratio by dry heating

at 50 and 60° C and 50 and 80% RH for 24 and 48 h [98]. A positive relation was found between the decrease in the free amino groups and color intensity of the prepared conjugates. Lowest color change was for conjugates from 1:2 protein: polysaccharide ratio. The temperature and RH were the main factors affecting the changes in the properties of the conjugates. Conjugation shifted the lowest solubility from pH 5 recorded for heated WPI to pH 4-4.5 for the conjugate and increased the foaming capacity of WPI. Optimum conditions for the preparation of glycated whey proteins of improved functional properties with the least changes in color, minimal loss of free amino groups (11%), and lysine (0.45%) were found by heating at 60° C and 0.49 a_W for 96 h of a 2:1 ratio of WPI to maltodextrin [99]. Around the pI of whey protein, the glycated WPI lost (~8%) of its solubility compared to ~60% for heated WPI. Also, glycated WPI showed twice thermal stability and 12% improvement in emulsion capacity than WPI, but it exhibited less emulsion stability [99]. The developed WPI-maltodextrin conjugate was recommended for use in high protein beverages of acceptable acidity (pH 4.5), longer shelf life which avoids the noticeable acidity and astringency of the commercial whey protein beverages. Aqueous solutions (pH 8.2) containing 5% from each WPI and maltodextrin corn steep syrup were heated at 90° C [100]. Maximum protein/saccharides conjugation with the lowest color change was obtained after heating for 8 h. Conjugation increased the solubility of WPI at pH 4.5 and its heat stability at 85° C depending on the dextrose equivalent of the used starch hydrolyzates. Conjugation of WPI and Maltodextrin (dextrose equivalent 6) by dry heating increased the protein solubility at pH 4.5, compared to the unheated and heated WPI solutions

[101]. Also, it enhanced the stability and clarity of the conjugate solution heated at 85°C for 10 min with 50 mM added NaCl.

Conjugation of WPI with low methoxyl pectin (SBP) through covalent interaction was achieved by dry heating at 60°C and 79% RH for 72 h [102,103]. The WPI: SBP ratio affected the solubility of the conjugate whereas the 3:1 and 2:1 ratios resulted in high increase in the solubility of WPI. Also, the conjugate made by the lowest SBP (3:1) was most effective in stabilizing oil/water emulsions. The heat stability of the conjugate was improved at all levels of SBP used as indicated by changes in the secondary and tertiary structure of WPI. Formation of whey protein-citrus pectin conjugates was carried out by extrusion processing [104]. The processing conditions were found to affect the obtained conjugate. The optimum formation of conjugates was obtained using reverse elements and the barrel temperature of 140ºC. The temperature at the die and the specific mechanical energy were of significant influence on conjugate formation and degradation.

The functional properties of WPI-chitosan conjugate prepared by dry heating at 79% RH were variably affected by the reaction conditions [105]. The solubility and heat stability of the conjugates were mainly affected by the protein: saccharide ratio, while the emulsion capacity and stability were mainly affected by the heating temperature [105].

Lactose glycated whey protein was prepared in nano fibrils form by heating WPI and lactose at pH 2 [106]. This preparation was highly dispersible in aqueous solutions, and remained transparent after heating at pH 3.0–7.0 and 0–150 mM NaCl. This has been attributed to the steric hindrance resulted from the presence of lactose at the surface of the formed nano fibrils which enable the dispersability and thermal stability of the conjugate.

The foaming, emulsifying and antioxidant properties of WPI glycated with the rare ketohexoses allose and D-psicose by dry heating were compared to that of WPI glycated with glucose and fructose [107]. WPI glycated with the allose and psicose showed higher emulsifying capacity, foam stability and antioxidant activity than those glycated with the aldohexose. The use of WPI glycated with allose and psicose in the manufacture of ice cream resulted in a product of improved quality and high antioxidant activity.

Glycation of whey proteins (WPs) with arabinose was significantly increased by ultrasound in the presence of polyethylene glycol as crowding agent [65]. Slight changes were only observed in the structure of WPs with an increase in glycation efficiency. Macromolecular crowding seems to intensify the oxidative modifications of WPs, and the formation of amyloid-like structures. The thermal stability, solubility at different pH, and antioxidant capacity of glycated WP were increased, especially in the presence of crowding agent, compared to sonicated non-glycated proteins.

5.2. Applications of glycated whey proteins.

The application of Maillard reaction products of food proteins including whey proteins has received much attention as an inexpensive and excellent source to emulsifiers and wall material for micro and nano-encapsulation of bioactive ingredients and probiotics [108]. WPI-maltodextrin conjugates were reported to exhibit excellent emulsifying properties. Fan *et al.,* [109] prepared β-carotene (BC) nano-emulsions stabilized with native WPI and Maillard reaction conjugates of WPI-DX (5 kDa, 20 kDa and 70 kDa) conjugates. The mean particle diameter of BC-loaded nano-emulsions stabilized with WPI-DX conjugates were significantly lower than that stabilized with native WPI. Remarkable pH stability of BC-loaded nano-emulsions was achieved by

using WPI-DX conjugates up to 30 d of storage at 25 and 50 °C. Also, encapsulation in nano-emulsions stabilized with WPI-DX conjugates inhibited lipolysis and release of BC.

Lui *et al.* [110] prepared high molecular weight Maillard reaction products from WPI and isomaltooligosaccharide (4:1) by wet heating at 90 °C for 4 h. The obtained conjugates (WIMRPs) were used to microencapsulate *Lactobacillus rhamnosus* (*L. rhamnosus*) by emulsification, and cold gelation. The WIMRPs microspheres showed higher encapsulation yield (88.88%) compared with the microcapsules prepared from mixed whey protein and isomaltooligosaccharide (WIMIX). WIMRPs microparticles were more spherical and smooth than the WIMIX microparticles. The WIMRPs and WIMIX microparticles had mean diameter of 183 μm and 201 μm respectively. WIMRPs provided better protection for probiotics after exposure to simulated gastrointestinal conditions and the probiotic were completely released within 60 min. Encapsulation of *L. rhamnosus* in the Maillard reaction conjugates of whey protein and isomaltooligosaccharide improved significantly its survival in white brined cheese during storage as compared to the free *L. rhamnosus* [111]. Also, it had no significant effect on the quality of the cheese.

Choi *et al.* [112] prepared Maillard reaction products (MRP) from mixtures of WPC and maltodextrin (1:2 and 1:3) and mixtures of WPI and maltodextrin (1:10 and 1:15). These conjugates were used to encapsulate conjugated linoleic acid (CLA) by emulsification and spray drying. The CLA encapsulated in WPI-based MRPs had smaller particle size and showed better encapsulation efficiency, and water solubility than those entrapped in WPI, WPC, or MD alone.

Eugenol was encapsulated in Maillard reaction conjugates of whey protein isolate (WPI) and maltodextrins (MD) by emulsification followed by spray drying [113]. The encapsulation efficiency was as high as 35.7 g/100 g eugenol, and capsules demonstrated transparent dispersions at pH 3.0 and 7.0 after heating at 80 °C for 15 min which indicates mean diameters smaller than 100 nm. The same wall material and method have been used to encapsulate thymol [114]. Dissolution of the obtained capsules gave a transparent and heat stable nano-dispersion containing concentrations of thymol above its solubility limit. These studies clearly indicate the feasibility of WPI-maltodextrin conjugates to encapsulate bioactive ingredients and probiotic. Conjugates of WPI with other polysaccharides need to be tested as encapsulants.

6. Conclusions and Future Trends.

As globular proteins whey proteins undergo several conformational changes and interactions during thermal treatments of milk products and in model systems. Glycation of whey protein during heat treatments has received special attention due to its effect on the technological and nutritional properties of milk and whey protein products. The extent of these changes depends on the severity of the heat treatments, heating conditions, medium and type and ratio of the carbohydrate present. In the presence of lactose as the main sugar present in milk products, lactosylation of the main whey proteins results in variable losses in lysine, decreases in their antigenicity and digestibility. Generally, the solubility and thermal stability of β-Lg and α-La can be improved by glycation because the pI of the protein is shifted to lower pH values and to the decrease in the formation of aggregates. Also, glycated β-Lg and α-La exhibit better emulsion stability, foam stability and high antioxidant activity than the native proteins.

Based on studies on individual whey proteins, attempts have been made to expand the applications of whey protein products particularly WPI via modification with

Maillard reaction. The use of polysaccharides particularly dextrans and maltodextrins for glycation and limiting glycation to the early stage of Maillard reaction aimed to produce conjugates modified with functional properties and least losses in the sensory and nutritional properties of the native proteins. Encouraging results have been obtained from several studies done along this line but more studies are needed to optimize the reaction conditions.

The nutritional and health effects of whey protein-polysaccharides conjugates need extensive research. Recent studies on glucose-WPI and glucose-WPC conjugates [115,116] suggest that these conjugates have potential health effects depending on the severity of the heat treatment used in their preparation. Early stage (EGPs) and advanced glycation (AGEs) products from Maillard reaction of WPI-glucose were reported to promote prostate cancer (PCa) proliferation in different ways [115]. The EGPs indirectly promote PCa through enhancement of cytokines formation by macrophages and in turn increase PCa cell proliferation, while AGEs increase directly PCa proliferation. Glucose-WPC conjugates [116] demonstrated immunomodulatory effects and increased phagocytic activity of RAW264.7 cells compared to WPC suggesting that this conjugates can be utilized to improve dietary source by enhancing immune modulating activity.

Exploiting the industrial uses of the developed glycated whey proteins is an area that needs further studies. As an example, the use of conjugated whey proteins in high protein beverages has been suggested for better product acceptability and extended shelf life but this needs to be confirmed experimentally. The WPI-DX and WPI-maltodextrin conjugates are characterized by excellent emulsifying properties which can be used as a new generation of emulsifiers in food systems. The use of WPI-polysaccharides as

prebiotics, natural antioxidants, stabilizers and emulsifiers needs to be explored in food products. Also, these conjugates can be used as wall materials for the encapsulation of bioactive ingredients, and microorganisms. Interest for edible coating for food products is an area of growing interest whereas the Maillard reaction products of whey proteins can play an important role. However, there still a need to optimize the process to obtain Maillard reaction products from whey proteins of the desired functional properties without the need for the removal of the un-reacted constituents.

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Fig 1: Steps of Maillard reaction (modified from [3]

Table 1: Changes in the functional properties of β-Lg subjected to Maillard reaction under different conditions using different reducing carbohydrates

Property	Carbohydrate	Reaction conditions	Results	Reference
Solubility	6 sugars	6 d/60°C, pH 5	35% less for ribose-β-Lg	[49]
	Dextran (10-20 KDa)	Dry heating/60°C, pH 7, 44% RH (24-72 hr)	Improved solubility 40% at pH 5 –decreased solubility at pH 4	[50,51]
Emulsification	Dextran	60 °C at an RH of 35–40% , 3 wk	Increased emulsion stability compared to hrated β-Lg	[52]
	Dextran of different MW	Dry heating, removal of unreacted dextran	Increased emulsion stability with increase in MW up to 150 kDa	[53]
	6 sugars	6 d/60°C, pH 5	Better emulsification with ribose and arabinose	[49]
	Dextran of different MW	60°C, 76% RH	Increased emulsion stability, increased thickness of adsorbed layer with higher MW dextran	[54]
	Dextran	60°C, 79% RH, 24 h	Increased emulsion stability and digestability of emulsified ol	[55]
	Fructo-oligosaccharides	Dry heating 50°C/24 hr, 79% relative humidity	Twice increase of emulsion stability	[43]
	4 sugars	Dry heating	Improved dispersion stability of emulsion	[56]
	Chitosan (different MW)	Dry heating 7 d/40°C, pH 6	Increased emulsion stability	[57]
Foaming	6 sugars	6 d/60°C, pH 5	Better foaming with glucose and galactose	[49]
	Lactose	Dry heating/ aqueous system	Increased adsorption and rigidity at air/water interface Dry>aqueous	[58]
	Glucose, lactose	Dry heating 50°C /relative humidity 65%	Increased foam stability, lactose -β-Lg conjugate gave more	[39]

			stable foam than glucose- β-Lg	
	Galactose	50°C/24-hr at pH 5, 7	Increased interfacial tension, foam capacity and stability heated at 5 but not at 7	[59]
	glucose	Dry heating 50°C/65% RH	Improved foam capacity	[60]
	galactose	50°C/24-hr followed by limited hydrolysis	Marked increase in foam stability	[61]
Thermostability	6 sugars	6 d/60°C, pH 5	Better heat stability	[49]
	Lactose, maltodextrin	Dry heating 130°C, 79% relative humidity	Increased heat stability after 20 min for lactose and 30 min for maltodextrin	[62]
	Ribose, glucose, maltotriose, fructose,maltose, galactronic acid	Dry heating 45°C, aw 0.53	Increased stability but more with size and negative charge	[63]
	Dextran	Dry heating 60°C, aw 0.44	Lower stability at pH 7.0, Higher stability at pH 5 and 85°C than native β-Lg	[50]
Antioxidant activity	6 sugars	6 d/60°C, pH 5	High scavenging activity, and 60 80% DPHH for arabinose and ribose	[49]
	Ribose	95°C/5 h	High antioxidant activity	[64]
	Glucose, ribose, galactose, lactose, arabinose	High intensity ultrasound assisted	Increased antioxidant activity, highest with ribose	[41]
	Arabinose	Ultrasound in the presence of ethylene glycol (crowding agent)	Increased antioxidant activity	[65]
	Chitosan (different MW)	Dry heating 7 d/40°C, pH 6	High ferric reducing power, highest with 1.3 kDa chitosan	[57]

Table 2: Changes in the functional properties of α- La subjected to Maillard reaction under different conditions using different reducing carbohydrates

Property	Carbohydrate	Reaction conditions	Results	Reference
Solubility	Dextran (10-20 KDa)	Dry heating/60°C, pH 7, 44% RH (24-72 h)	Improved solubility 5-50% at pH 3.5- 5 highest at pH 4	[17]
Emulsification	Lactose	High pressure treatment followed by dry heating 50°C, 44% RH, 168 h	Improved emulsifying properties after high pressure	[84]
Foaming	Rhamnose, fucose, glcose, galactronic acid, oligosaccharides	Dry heating	Glycation with rhamnose and fucose increased foam stability	[76]
Thermostability	Dextran (10-20 KDa)	Dry heating/60°C, pH 7, 44% RH (24-72 h)	Higher stability of conjugated α- La than the heated α- La	[17]
Antioxidant activity	Allose, glucose, fructose	Dry heating/50°C, 59% RH (48 h)	Allose induce faster glycation and higher actioxidant activity	[85]
	Ribose	95°C/5 h	High antioxidant activity	[64]

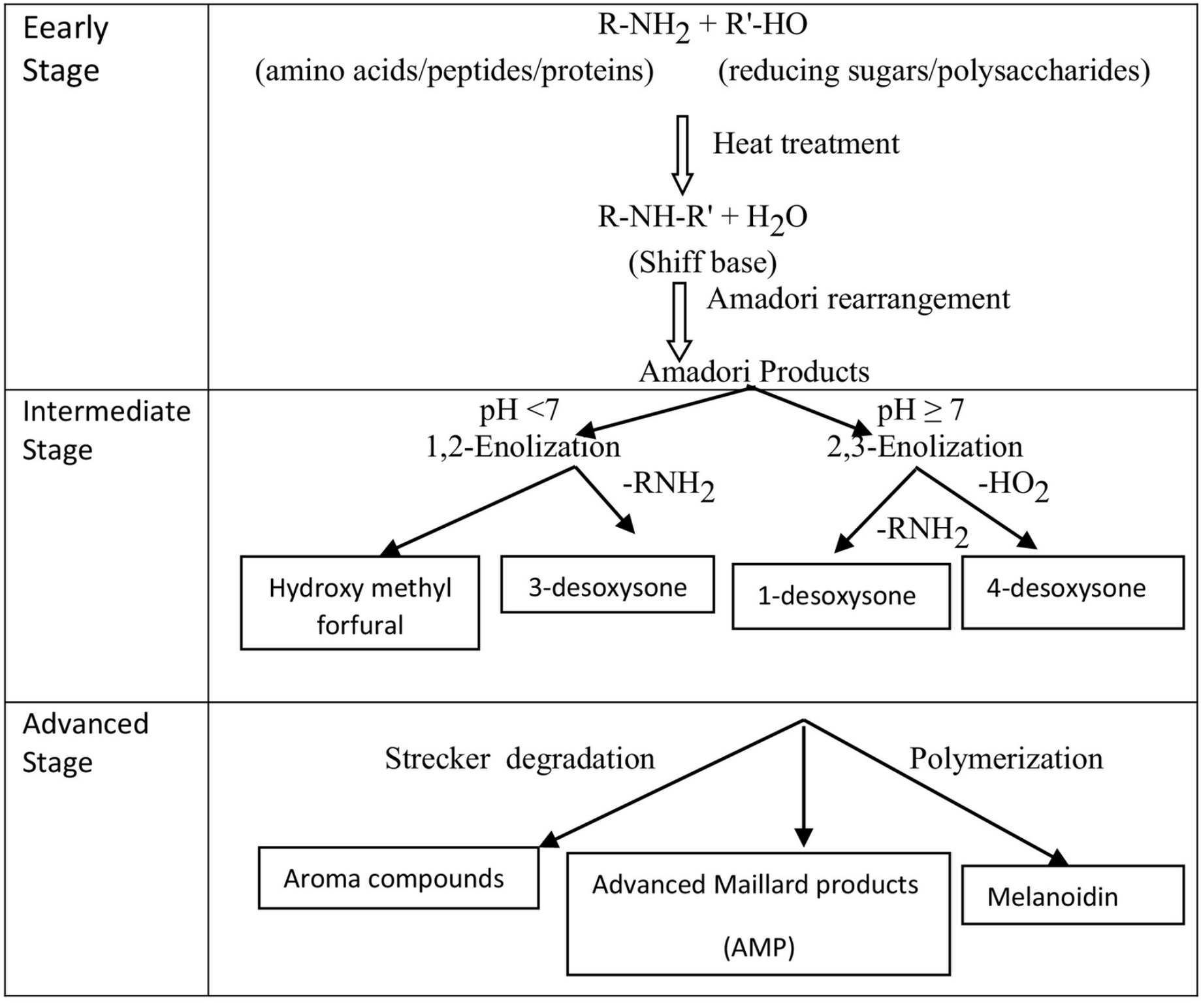
Eearly Stage
R-NH2 + R'-HO
(amino acids/peptides/proteins)
(reducing sugars/polysaccharides)
Heat treatment
R-NH-R' + H2O
(Shiff base)
Amadori rearrangement
Amadori Products
Intermediate Stage
pH <7
1,2-Enolization
-RNH2
pH ≥ 7
2,3-Enolization
-HO2
-RNH2
Hydroxy methyl forfural
3-desoxysone
1-desoxysone
4-desoxysone
Advanced Stage
Strecker degradation
Polymerization
Aroma compounds
Advanced Maillard products
(AMP)
Melanoidin

Figure 1